THE FORMATION OF 5S RIBOSOMAL RIBONUCLEIC ACID IN BACILLUS SUBTILIS BY POSTTRANSCRIPTIONAL MODIFICATION

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In addition to the 16*S* and 23*S* species of RNA, the bacterial ribosome has been shown to contain a much smaller component RNA called 5*S*, having a molecular weight of the order of 40,000 daltons.[4–6, 15] This small rRNA, like the others, exists in the proportion of one molecule per 70*S* ribosome, and it is found associated with the 50*S* ribosomal subunit.[5] The ribosomes of all organisms so far examined contain this small type of RNA in addition to the two larger types. Our understanding of the 5*S* rRNA molecule is at present rather limited. It is known through sequence analysis and molecular hybridization techniques that 5*S* rRNA possesses a unique primary structure and one having no detectable sequence homologies with its larger counterparts.[3, 12] The 5*S* rRNA cistrons (in *Bacillus subtilis* at least) are located in the same region of the genome that contains the 16*S* and 23*S* rRNA cistrons as well as (some or all of the) tRNA cistrons.[12] However, the details of 5*S* biosynthesis and function are for the most part an unsettled matter. The present report is a study of 5*S* rRNA biosynthesis in *Bacillus subtilis*. We show that this rRNA, like its mature 16*S* and 23*S* counterparts, is not a so-called "primary transcription product," but arises by posttranscriptional modification of some primary transcription product, a modification that apparently involves its cleavage from a larger precursor RNA.

Materials and Methods.—All the materials and methods used herein have been described in detail previously.[1, 7, 8] The W23 strain of *B. subtilis* was used throughout. Cells were grown aerobically at 37°C in a salts-amino acids medium. In labeling experiments cell growth was stopped abruptly by pouring cultures onto ice-azide mixtures. Cells were harvested by centrifugation, frozen cell pellets were ground with glass beads, and RNA was extracted by a phenol procedure.[7, 8] In all experiments RNA carried a double radioactive label—H^3-uridine as a long-term (>2 cell generations) label, and C^{14}-uridine as the "pulse" label.[8] RNA analysis was by polyacrylamide gel electrophoretic techniques, a 6% acrylamide gel being employed where the 5*S* rRNA was concerned, a 2.4% gel where the 16*S* and 23*S* rRNA's were concerned.[1, 8]

Results.—The mature forms of the 16*S* and 23*S* rRNAs (called m16 and m23) that exist in the completed 30*S* and 50*S* ribosomal subunits appear to differ from their "immature" precursors (called p16 and p23) found in the so-called "eosomal" and "neosomal" stages of ribosome development, in that the precursors each appear to be from 5 to 20 per cent larger than their mature counterparts.[8] Thus production of m16 and m23 seems to involve a posttranscriptional modification (presumably cleavage) of preexisting RNA's. The third-order kinetics seen for the initial appearance of radioactive label into m16 and m23 is consistent with this view. On the other hand, the precursors, p16 and p23, do appear to be primary transcription products, as judged by the first-order kinetics of the initial appearance of labeled uridine into them.[8]

Two other characteristics of m16 and m23 useful in characterizing them as being formed by posttranscriptional modification are: (1) that chloramphenicol (CM), which seems to have little or no effect on the transcription process, does, however, prevent the conversion of p16 into m16 or of p23 into m23,[8, 13] and (2) that p16 and p23 syntheses are prevented by actinomycin D (AMD), a general transcription-blocking agent, but the p16 → m16 and p23 → m23 conversions are not.[2, 9] In all the above ways the formation of 5*S* rRNA mimics that of its m16 and m23 counterparts, as we shall now see.

Figure 1 demonstrates that polyacrylamide gel electrophoresis can give a complete separation between the 5*S* rRNA and tRNA species from *B. subtilis*—confirming a fact already established for *E. coli* RNA.[1] The peak called 5*S* rRNA can be identified by the fact that this species is extractable from ribosomes.

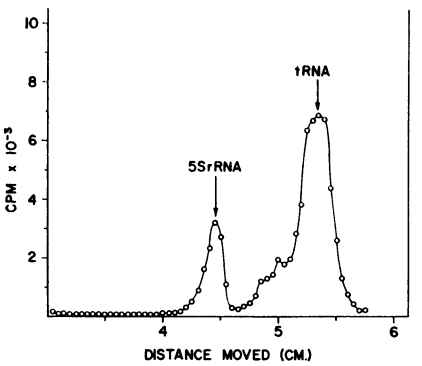

FIG. 1.—Separation of *Bacillus subtilis* 5*S* rRNA from tRNA by polyacrylamide electrophoresis. A log-phase culture of *B. subtilis* has been exposed to H^3-uridine (~4 μc/ml) for two generations. Whole-cell RNA has been extracted as described and analyzed by electrophoresis on a 6% polyacrylamide gel. *Ordinate,* cpm H^3 in a slice; *abscissa,* position of slice in gel, i.e., the distance the RNA has moved.

Figure 2 presents the kinetics of incorporation of labeled uridine into various RNA species. Incorporation is expressed as the ratio of the counts in the C^{14}-pulse to the counts in the H^3 long-term label, the latter, which are nearly constant over the time interval shown, serving as a measure of total cell RNA. Details of this kind of experiment have been reported previously.[8] Initial first-order kinetics (slope 1.0 on a log-log plot) are shown by total cell RNA (predominantly some mixture of message RNA and ribosomal precursor RNA's) and by isolated p16 and p23 (the latter not shown in the figure). Initial higher-order kinetics (a slope of ~3 on the log-log plot) are shown by m16 and 5*S* rRNA. Initial first-order kinetics are consistent with an RNA as a primary transcription product.[8, 10, 11] Initial higher-order kinetics preclude an RNA as a primary transcription product; such RNA's must pass through one or more macromolecular intermediate stages before arriving at their characteristic form.[8, 10, 11]

Figure 3 shows that chloramphenicol (CM) prevents not only the formation of m16, as was known,[8, 13] but that of 5*S* rRNA as well. In this case a culture treated with CM was given a two-minute pulse of C^{14}-uridine, the pulse being terminated by addition of a large excess of C^{12}-uridine (*see* Fig. 3 legend for details). (Although a swamping amount of unlabeled uridine is known not to halt immediately the uptake of labeled uridine,[9] this fact in no way alters the pertinent conclusions that can be drawn.) It is clear that, although significant amounts of labeled p16 have been formed in the two-minute pulse interval,

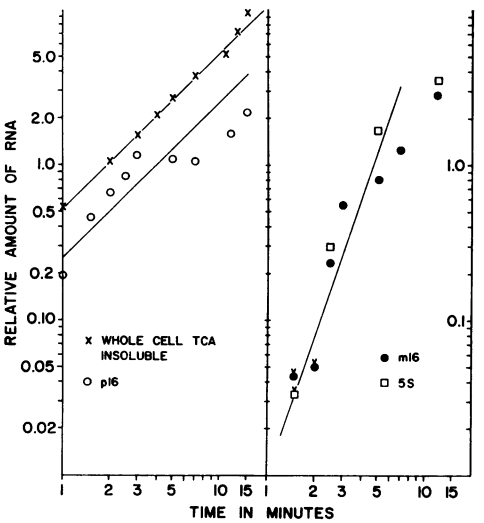

FIG. 2.—Kinetics of incorporation of labeled uridine into various RNA fractions. A log-phase culture of *B. subtilis* carrying a long-term H^3-uridine marker is labeled for varying times with C^{14}-uridine;[8] the C^{14}:H^3 ratios for various RNA fractions (in arbitrary units) are plotted as a function of incorporation time. (However, the whole-cell trichloracetic acid-insoluble fraction is plotted merely as total counts per minute incorporated in arbitrary units—actual counts per minute ranging from 5×10^3 to 100×10^3.) A caret over a point indicates an upper bound. Slope of left-hand lines is 1.0; slope of right-hand line is 3.0. [In calculating the C^{14}:H^3 ratios correction for nonspecific "background"—i.e., mRNA—has been made as described previously.[8] The H^3 count in the m16 peak was used in calculating the C^{14}:H^3 ratios for p16.[8]]

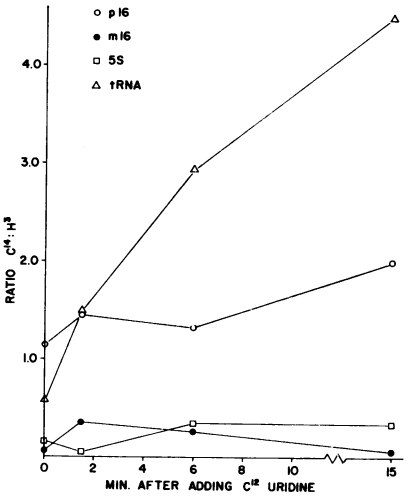

FIG. 3.—Effect of chloramphenicol on synthesis of 5*S* rRNA. A log-phase culture of *B. subtilis*, carrying a long-term H^3-uridine label, is treated with CM (final conc. 200 μg/ml). Five min later a C^{14}-uridine "pulse" is begun. After 2 min, C^{12}-uridine (final conc. 1 mg/ml) is added. At that time and thereafter as a function of time, portions of the culture are removed, and the RNA extracted and analyzed by polyacrylamide gel electrophoresis as described. The "specific activity" in each fraction (see Fig. 2 caption) is plotted as a function of time after addition of C^{12}-uridine.

negligible amounts of label can be "chased" into either m16 or 5*S*, rRNA's, although label does continue to appear in tRNA, as expected. A parallel control culture, untreated with CM, behaved as expected—m16, m23, 5*S* rRNA, and tRNA continued to incorporate labeled uridine after the onset of the "chase" (all these RNA's having approximately equal "specific activities" at any given time), while p16 and p23 levels of radioactivity dropped as a function of "chase" time.

The parallels between 5*S* rRNA and m16 formation strongly suggest the former, like the latter, to be derived from a macromolecular precursor. If the hypothetical 5*S* precursor were to accumulate in the presence of CM, it should be possible to demonstrate its existence by showing its conversion to 5*S* rRNA (upon removal of CM) in the absence of transcription. Table 1 shows the results of an experiment of this type. CM has here been added to a log-phase culture of *B. subtilis* (again carrying a long-term H^3-uridine label). Subse-

TABLE 1. *Formation of 5S rRNA in the presence of actinomycin D.*

	(*A*) 5*S* rRNA	(*B*) tRNA	(*C*) (*A*)/(*B*)
Control—stopped at <1-min incubation	0.23	1.4	0.16
Exptl.—incubated in AMD medium for 10 min	0.57	1.3	0.44

A 400-ml log-phase culture of *B. subtilis* prelabeled with 100 μc H^3-uridine (for more than two generations) was treated with CM (final conc. 200 μg/ml). After 5 min, 20 μc C^{14}-uridine were added and the culture aerated for another 20 min. Cells were then rapidly cooled to 0°C, harvested by centrifugation, and washed twice in ice-cold nutrient broth medium. The final cell pellet was resuspended in a small volume of cold medium and then added to 400 ml of nutrient broth, at 37°C, containing 10 μg/ml of AMD. One half of the culture was then harvested immediately (i.e., in <1 min) and processed, as described under *Materials and Methods* for RNA. The remaining half was harvested after a 10-min aeration in the AMD medium. The resulting RNA's were analyzed in the customary fashion on 6% polyacrylamide gels. Column (*A*) shows the specific activity (C^{14}:H^3 ratio) in the 5*S* peak for the two halves of the culture, column (*B*) the specific activity for the tRNA peak, and column (*C*) the ratio of column (*A*) to column (*B*).

quently, C^{14}-uridine has been added and the culture allowed to incubate for some time. The cells were then collected by centrifugation, washed free of CM, and finally introduced into a prewarmed medium (nutrient broth) containing actinomycin D (AMD)—see table caption for details. One half of the resulting culture was harvested within the first minute of incubation, the remaining half after ten minutes of incubation at 37°C. Although no new RNA synthesis can occur in the AMD medium,[14] as can be seen also from the tRNA fraction in the table (and as confirmed by appropriate controls), still appreciable amounts of labeled 5*S* material accumulate during incubation in the AMD medium. In addition, the p16 and p23 formed in CM medium do, of course, break down during the subsequent incubation, but whether the m16 and m23 formed in this instance are entirely normal is presently in doubt and under investigation. If we take the C^{14}:H^3 ratio for tRNA as a measure of the amount of any type of RNA (or its precursor) made during incubation in CM medium, then we see that about 40 per cent of the theoretical maximum amount of the 5*S* rRNA precursor assumed to be made in CM medium has been converted to 5*S* rRNA in the ten-minute incubation in the AMD medium. (We cannot explain in a unique way the smaller amount of 5*S* rRNA present in the "zero" time control, but there are a number of trivial explanations possible for it, and its existence—particularly in view of the data in Fig. 3—in no way alters the main conclusion we draw.)

Discussion.—The above results make it clear that in *Bacillus subtilis* the so-called 5*S* rRNA is not a primary transcription product. Its formation necessitates the posttranscriptional modification of some pre-existing RNA. The supporting evidence is threefold: (1) The incorporation of labeled uridine into 5*S* rRNA initially follows a "higher-order" kinetics (as does incorporation into m16 and m23), while first-order kinetics are obeyed for incorporation into known precursor RNA's such as p16 and p23. [Galibert *et al.*[6] have also reported that incorporation of label into 5*S* rRNA does not show an initial first-order kinetics. However, we feel that the measurement in this case is merely an artifact, not a true determination of RNA synthesis kinetics. In order to prove that incorporation kinetics reflect RNA synthesis kinetics, it is at least necessary to show: (*a*) that some RNA species (i.e., a primary transcription product) does manifest initial first-order incorporation kinetics, or (*b*) that any apparent higher-order

kinetics are not merely due to the label's equilibrating with pools of small, molecular weight intermediates. Neither of these points has been demonstrated by Galibert *et al.* Actually it *is* known that the kinetics of incorporation of P^{32} (the isotope used by these workers) into acid-insoluble products show a *lag* (i.e., would appear as higher-order kinetics), and that this is not true of uracil incorporation,[11] the latter fact being confirmed in the above studies. This fact alone is undoubtedly the explanation for the 5*S* rRNA kinetics reported by Galibert *et al.*, and also explains their report that 5*S* rRNA and 16*S* and 23S rRNA all show the *same* kinetics of incorporation. Their 16*S* (or 23*S*) rRNA fraction contains both p16 and m16 (or p23 and m23), and so, if their incorporation kinetics were measuring true RNA synthesis kinetics, incorporation into their 16*S* and 23*S* fractions *should* have been first-order initially.] (2) CM (which does not prevent transcription) does prevent the formation of 5*S* rRNA; and (3) in the presence of AMD concentrations that halt transcription completely, 5*S* rRNA can still be formed to some extent. What we have failed to do to date is to detect the presumed precursor of the 5*S* rRNA. We can say, however, that such a precursor is not a small RNA of molecular weight similar to that of the 5*S* rRNA, for no RNA peaks slightly larger than 5*S* rRNA are detected electrophoretically in pulse-labeled RNA preparations. (In this respect then, 5*S* rRNA does not resemble its m16 and m23 counterparts.)

A reasonable hypothesis for 5*S* rRNA origin is that it may come from one of the RNA segments split from p16 or p23 when m16 and m23 are formed. For the case of p23, the calculated molecular weight of the RNA presumably cleaved to produce m23 is of approximately the correct value to be the 5*S* rRNA—i.e., $\sim 5 \times 10^4$ daltons.[8] This is also consistent with the fact that the 5*S* rRNA appears on the 50*S* ribosomal subunit along with m23 at the time of the neosome → ribosome transition. This hypothesis, however, in its simplest form runs contrary to the assertion of Morell *et al.*[12] that the 5*S* and 23*S* rRNA cistrons are not located at the exact same point on the genome. However, we do not consider such an objection a compelling one, given the occasionally uncertain state of measurements made by nucleic acid hybridization.

We are attempting to resolve the origin of the 5*S* rRNA by competing the molecular hybridization of 5*S* rRNA by the various precursor rRNA's.

Summary.—The 5*S* ribonucleic acid (RNA) molecule, appearing as a component of the 50*S* ribosomal subunit, is not formed directly by transcription of a cistron(s). Instead, it appears to be formed through what is probably a primary structural modification of some preexisting RNA. Three lines of evidence lead to this conclusion: (1) the kinetics of incorporation of radioisotopes into the 5*S* rRNA, (2) the effect of chloramphenicol on its formation, and (3) the fact that some 5*S* rRNA formation can occur in the apparent absence of transcription.

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